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Interaction of FliS flagellar chaperone with flagellin

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Abstract Premature polymerization of flagellin (FliC), the main component of flagellar filaments, is prevented by the FliS chaperone in the cytosol. Interaction of FliS with flagellin was characterized by isothermal titration calorimetry producing an association constant of 1.9×10^7 M^{-1} and a binding stoichiometry of 1:1. Experiments with truncated FliC fragments demonstrated that the C-terminal disordered region of flagellin is essential for FliS binding. As revealed by thermal unfolding experiments, FliS does not function as an antifolding factor keeping flagellin in a secretion-competent conformation. Instead, FliS binding facilitates the formation of α-helical secondary structure in the chaperone binding region of flagellin.

Keywords: FliS; Flagellar chaperone; Flagellin; Flagellar export; Type-III export

1. Introduction

Bacteria swim by rotating flagellar filaments, each of which has a helical shape and works as a propeller [1]. External flagellar proteins, lying beyond the cytoplasmic membrane, are synthesized in the cell and exported by the flagellum-specific export apparatus from the cytoplasm to the site of assembly through the central channel of the flagellar filament. The flagellum-specific export system is a specialized type III export machinery [2].

Little is known about the export process of flagellar proteins through the hollow core of the flagellum to their site of polymerization. Owing to the small diameter of the export channel (25–30 Å) [3], proteins destined for incorporation into the growing flagellum are thought to be exported in a partially unfolded state, implying that premature folding and oligomerization in the cytosol must be prevented. It seems that cells prevent the cytosolic folding and interaction of these proteins applying flagellar chaperones [4–6] that specifically interact with individual axial proteins and may keep them in a secretion-competent conformation.

FliS acts as a substrate-specific chaperone facilitating the export of flagellin (FliC) [6,7], the main component of flagellar filaments. Both terminal regions of flagellin, comprising 66 N-terminal and 44 C-terminal residues, are disordered in the monomeric form whereas the central part of the molecule is composed of three well-folded domains [8,9]. The highly conserved disordered regions are essential for self-assembly of flagellin [10]. They become stabilized into α-helical bundles upon polymerization forming the core part of filaments [3].

FliS from *Salmonella typhimurium* consists of 135 amino acid residues [11] and predicted to be predominantly α-helical [7]. The first crystallographic structure of a flagellar chaperone, *Aquifex aeolicus* FliS [12], demonstrated that FliS adopts a novel fold, clearly distinct from those of the type III secretion chaperones. FliS effectively inhibits in vitro polymerization of flagellin [7]. Yeast two-hybrid assays indicated that the C-terminal disordered region of flagellin is essential for FliS binding [13]. Gel filtration chromatographic experiments suggested that *Salmonella* FliS forms stable homodimers which bind to FliC monomers [7]. Yet, the structure of *A. aeolicus* FliS in complex with a C-terminal fragment of flagellin shows a monomer to monomer interaction [12].

Here we addressed the mechanism of *Salmonella* FliS chaperone action. FliS–FliC complex formation was quantitatively characterized by isothermal titration calorimetry (ITC) to determine binding stoichiometry. Conformational rearrangements accompanying binding of FliS to FliC were also studied. We aimed at answering the question whether chaperone binding has only a local effect on the binding region of flagellin, or transmitted to other parts of the molecule keeping flagellin subunits in an unfolded and secretion-competent state.

2. Materials and methods

2.1. Protein expression and purification

Flagellin was prepared from *S. typhimurium* wilde-type strain, SJW1103, and purified as described [8]. Fragments of flagellin missing terminal segments of various sizes within the disordered terminal regions were prepared by limited proteolysis of monomeric flagellin

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Abbreviations: FliC, flagellin; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; CD, circular dichroism

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and purified by ion-exchange column chromatography [10]. The F(59-494) fragment was produced by Endoproteinase Lys-C (ELC), F(1-461) was prepared by Endoproteinase Glu-C (V8). ELC and V8 were obtained from Boehringer (Roche). Other chemicals were of analytical grade from commercial sources.

FliS was overproduced in *Escherichia coli* cells, strain BL21(DE3), carrying pET-3c-based plasmids. FliS producing bacteria were kindly provided by Hideyuki Matsunami (Dynamic NanoMachine Project, ICORP, JST, Japan). 20 ml overnight culture was inocculated into 1000 ml of LB broth, supplemented with Ampicillin (50 μg/ml) and Chloramphenicol (30 μg/ml), and incubated at 37 °C to reach an $OD_{600\ nm}$ of 0.6. After addition of IPTG to a final concentration of 1 mM, incubation was continued for another 4 h. The bacterial pellet was collected by centrifugation at 4500 rpm for 20 min and stored at −30 °C.

Purification of FliS protein was performed as follows: The bacterial pellet from 2 L culture was resuspended in 30 ml PBS, sonicated on ice to lyse the cells for 1 min, 3 times at a power of 14 W (CP-130, Cole-Palmer Vernon Hills, IL), and the suspension was centrifuged at 12 000 × *g* for 30 min at 4 °C. The crude inclusion body pellet was fully resuspended in 15 ml of 3% Triton X-100, 50 mM Tris–HCl, 1 mM EDTA (pH 8.0) and sonicated on ice. The solution was centrifuged at 8000 × *g* for 25 min at 4 °C to remove membrane fragments and cell debris. The supernatant was collected and the pellet was washed again. This step was repeated 2 times with Triton X-100, and then 2–3 times without Triton X-100. The collected supernatants were centrifuged at 38 000 rpm for 30 min at 4 °C. The inclusion body pellet was washed 2 times with 50 mM Tris, 1 mM EDTA, pH 8. Then, the pellet was dissolved in 18 ml of 6 M Guanidine–HCl, 50 mM Tris, pH 8, 10 mM EDTA containing 2 Complete mini protease inhibitor cocktail tablets (Roche) and incubated overnight at 4 °C on a shaker. The sample was centrifuged at 38 000 rpm for 30 min at 16 °C, and the supernatant was used for final purification by FPLC on a HiPrep Sephacryl 16/60 S-200 gel filtration column (Pharmacia Biotech) applying 20 mM Tris, 150 mM NaCl, pH 7.8, buffer.

The protein concentration of samples was determined from absorption measurements at 280 nm using extinction coefficients calculated from the known aromatic amino acid contents of the molecules [14]. Purity of protein samples was checked by SDS–PAGE using 12.5% and 20% polyacrylamide gels, stained with Coomassie blue R-250 (Merck).

2.2. Scanning microcalorimetry

Differential scanning calorimetric experiments were performed with a VP-DSC instrument (MicroCal, Northampton, MA) interfaced to an IBM PC for automatic data collection and instrument control. Calorimetric measurements were done in 20 mM Tris–HCl buffer solution (pH 7.8) at a scanning rate of 1 °C/min. Protein samples were extensively dialyzed against the buffer at 4 °C. In the calculations of molar thermodynamic quantities, the molecular masses used were 16.5 kDa and 51.4 kDa for FliS and FliC, respectively. Differential scanning calorimetry (DSC) data were analyzed with an Origin 5.0-based software package supplied by MicroCal.

2.3. Isothermal titration calorimetry

The experiments were carried out with a VP-ITC titration calorimeter (MicroCal, Northampton, MA). Measurements were done in 20 mM Tris–HCl, pH 7.8, at various temperatures. Protein samples were extensively dialyzed against the buffer at 4 °C. All solutions were thoroughly degassed before use by stirring under vacuum. FliS solution was loaded into the calorimetric cell and 10 μl portions of concentrated FliC solution were injected. To take into account heats of dilution, two blank titrations were performed; one injecting FliC solution into buffer and another injecting buffer into the FliS solution. The heat released by dilution of FliC was negligible. The averaged heats of dilution were subtracted from the main experiment. Calorimetric data were analyzed using MicroCal Origin software fitting them to a single binding site model.

2.4. Circular dichroism (CD) measurements

Far-UV CD spectra were recorded in the range of 200–260 nm at 0.5 nm intervals on a Jasco-720 spectropolarimeter. Samples were measured at room temperature in cylindrical fused quartz cells with a path length of 0.1 cm in 10 mM phosphate buffer (pH 7.0).

3. Results

3.1. Thermodynamic characterization of FliC–FliS association

Isothermal titration calorimetry was used to determine directly the energetics and stoichiometry of FliS–FliC interaction. FliS solution at a protein concentration of 0.2 mg/ml was loaded into the calorimetric cell and 10 μl portions of concentrated FliC solution (c = 5.5 mg/ml) were injected. The observed heat effects clearly indicated the interaction (Fig. 1, upper panel). The measured data were analyzed and nicely fitted using a one-site model (Fig. 1, lower panel). A large and favorable enthalpy of binding ($\Delta H = -12.9$ kcal/mol) was observed upon interaction at 25 °C, accompanied by an unfavorable binding entropy ($\Delta S = -9.9$ cal mol^{-1} deg^{-1}). The FliS–FliC association had an equilibrium association constant of 1.9×10^7 M^{-1}. Our calorimetric data clearly show that FliS interacts with FliC with a 1:1 stoichiometry (N = 1.00). It is worth noting that significant interaction persisted between FliS and FliC even at 55 °C, well-above the unfolding temperature of flagellin (data not shown).

To localize the binding regions terminally truncated fragments of flagellin were prepared by proteolytic digestion. F(59-494) was deprived of its disordered N-terminal region, while F(1-461) lacked a large portion of the C-terminal

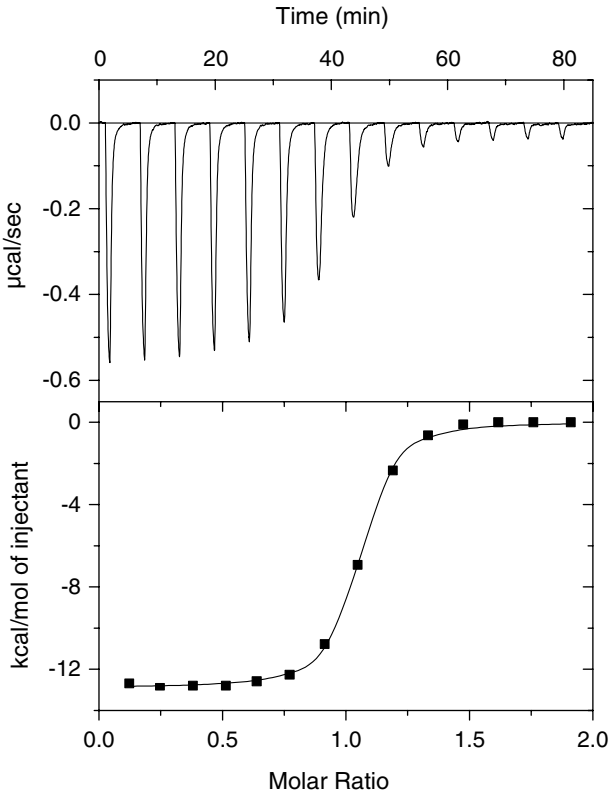

Fig. 1. Isothermal calorimetric titration of FliS with flagellin at 25 °C. Upper part: repetitive 10 μl injections of FliC (c = 5.5 mg/ml) into a FliS (c = 0.18 mg/ml) solution caused exothermic heat pulses. Lower part: changes in binding enthalpy (■) of the corresponding injections shown above as a function of the molar FliC to FliS ratio. The solid line is the least-squares fit of the data to a one binding site model resulting in the following parameters: stoichiometry N = 1.00, $K = 1.9 \times 10^7$ M^{-1}, $\Delta H = -12.9$ kcal/mol. Titrations were done in 20 mM Tris–HCl, pH 7.8, buffer.

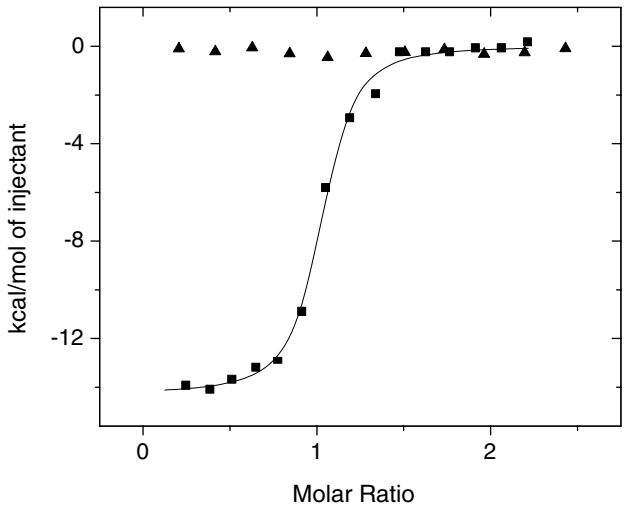

Fig. 2. Calorimetric binding isotherms for titration of FliS with truncated flagellin fragments of F(59-494) (■) and F(1-461) (▲). Concentrated F(59-494) (c = 5.5 mg/ml) or F(1-461) (c = 5.6 mg/ml) solutions were injected in 10 μl portions into a 0.19 mg/ml solution of FliS in 20 mM Tris–HCl buffer, pH 7.8, at 25 °C. The solid line represents the best fit for FliS-F(59-464) interaction with a one binding site model. (Best fit parameters: stoichiometry $N = 0.97$, $K = 1.0 \times 10^7$ M^{-1}, $\Delta H = -14.0$ kcal/mol.)

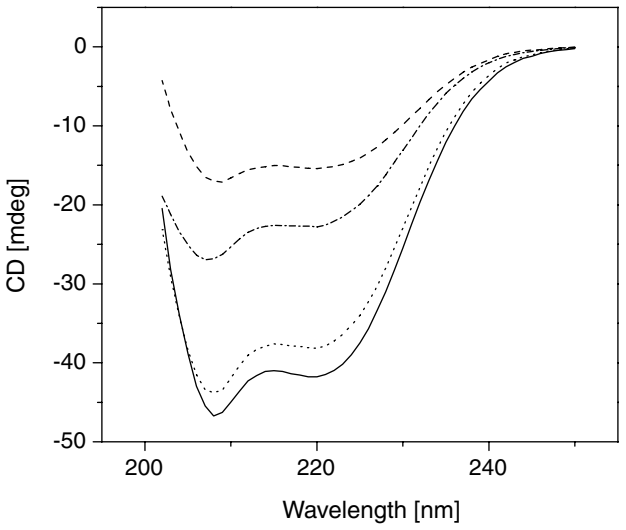

Fig. 3. Comparison of the CD spectra of FliS (dash), FliC (dash-dot) and FliS/FliC complex (solid) in the far-UV region. The dotted spectrum is the sum of the FliS and FliC spectra. Measurements were done in 10 mM phosphate buffer solution (pH 7.0) at protein concentrations of 0.13 mg/ml and 0.26 mg/ml for FliS and FliC, respectively.

disordered part. These fragments were produced by highly specific proteases [10], purified by ion-exchange chromatography and their interaction with FliS was examined by ITC (Fig. 2). FliS strongly bound to F(59-494) with an association constant ($K = 1.0 \times 10^7$ M^{-1}) and binding enthalpy (ΔH = 14.0 kcal/mol^{-1}) similar to that of the FliS–FliC interaction. However, the C-terminally truncated F(1-461) fragment completely lost FliS-binding ability. These results demonstrate that the C-terminal disordered region of flagellin plays a decisive role in FliS–FliC interaction.

3.2. Conformational rearrangements upon FliS–FliC interaction

Far-UV CD spectra of FliS, FliC and the FliS–FliC complex were recorded and compared to reveal possible conformational changes accompanying FliS–FliC interaction. Measurements were done in 10 mM phosphate buffer (pH 7.0) at protein concentrations of 0.13 mg/ml and 0.26 mg/ml for FliS and FliC, respectively. Both flagellin and FliS exhibited CD spectra characteristic for proteins with a significant α-helical structure (Fig. 3). FliS is a well-ordered protein predicted to contain about 70% α-helical structure (unpublished data). Although the terminal regions of flagellin are disordered in solution, the central portion of the amino acid sequence forms three ordered domains with significant (~24%) helical content [9].

Comparison of the sum of FliS and FliC spectra with the spectrum of the FliS–FliC complex shows a significant decrease of the signal around 222 nm indicating an α-helical ordering as a result of interaction (Fig. 3). Under the applied conditions FliC is highly complexed with FliS. From the amplitude of the spectrum at 222 nm, we can make a rough estimate for the number of residues involved in helical stabilization. Taken

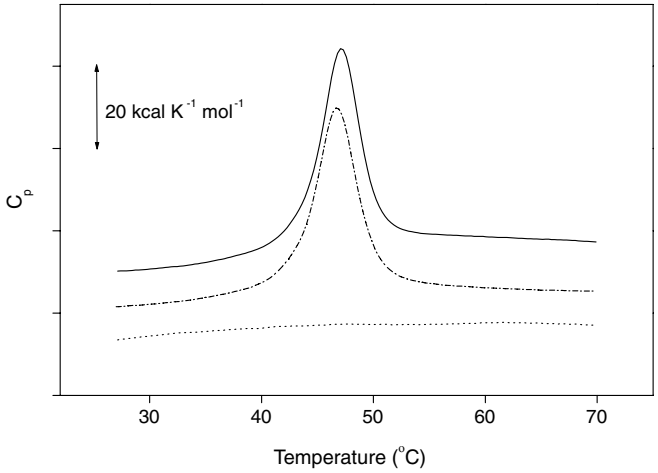

Fig. 4. Temparature induced unfolding of FliS (dot), FliC (dash-dot) and the FliS/FliC complex (solid). Calorimetric melting profiles were measured in 20 mM Tris–HCl (pH 7.8). The scanning rate was 1 °C/min. FliS does not show temperature induced unfolding in the 25–70 °C range.

the helix content to be directly proportional to the mean residue ellipticity at 222 nm [15], approximately 20 residues appear to adopt an α-helical structure as a result of FliS–FliC complex formation.

3.3. Effect of FliS binding on flagellin stability

Adiabatic scanning microcalorimetry was employed to reveal the effect of FliS binding on the structural stability of flagellin. Measurements were performed at protein concentrations of 0.4 mg/ml and 1.0 mg/ml for FliS and FliC, respectively. Under these conditions flagellin is almost fully complexed to the chaperone as indicated by ITC measurements. Melting profiles of FliS, FliC and FliS/FliC samples were obtained by DSC in the 20–70 °C region, and presented in Fig. 4.

Interestingly, FliS alone did not exhibit any unfolding transition in the studied temperature range. FliS underwent a temperature induced denaturation accompanied by strong precipitation above 100 °C (data not shown). Flagellin unfolded around 47 °C, as was reported earlier [9]. Its unfolding curve is dominated by thermal denaturation of the central compact part of the molecule because the disordered terminal regions of flagellin have no significant contribution to the melting process [9]. The FliS/FliC sample exhibited a very similar heat-denaturation curve to that of flagellin. The transition midpoint temperatures were almost the same: 46.7 °C and 47.1 °C for FliC and FliS/FliC, respectively. Sequential heating experiments revealed that thermal denaturation of both samples was highly reversible. The area below the heat absorption peak corresponds to the enthalpy of protein denaturation at the transition temperature. Melting transitions were accompanied by an enthalpy change of 248 kcal/mol and 232 kcal/mol for FliC and FliS/FliC, respectively. The similar transitional enthalpies of flagellin and FliS/FliC indicate that binding of FliS to FliC does not significantly influence the stability of the folded domains of flagellin.

4. Discussion

Axial components of bacterial flagellum are synthesized in the cytoplasm, but most of them fulfill their role outside the cell and are exported by the flagellum-specific export system through the cell membrane. Flagellar chaperones are known to help the export process [2,16]. FliS is a flagellar chaperone specific for flagellin. It prevents premature polymerization of flagellin subunits in the cytosol [7]. As shown by our ITC experiments, the FliS–FliC association is strong ($K = 1.9 \times 10^7\ M^{-1}$) and FliS interacts with FliC in a 1:1 stoichiometry. Although previous observations by gel filtration chromatography suggested that a FliS dimer interacts with a FliC monomer [7], the structure of *A. aeolicus* FliS in complex with a C-terminal fragment of flagellin also shows a monomer to monomer interaction [12]. Experiments using yeast two hybrid mutant system indicated [13] that the C-terminal disordered part of flagellin plays and essential role in FliS–FliC interaction. This observation was confirmed in this study by direct binding experiments with terminally truncated fragments of flagellin.

Our ITC measurements revealed a significant entropy decrease upon FliS–FliC complex formation suggesting that the interaction is accompanied by conformational stabilization. Indeed, secondary structure formation was revealed by CD spectroscopy: about 20 residues were estimated to adopt an α-helical structure as a result of interaction. While FliS has a well-ordered tertiary structure, terminal regions of flagellin are disordered in solution. These terminal regions have a high α-helix-forming potential, and they form helical bundles upon polymerization [3,9]. Since the disordered C-terminal portion of flagellin is involved in the interaction with FliS [13, this study], it is reasonable to assume that this disordered segment becomes stabilized into an α-helical conformation upon FliS interaction.

The diameter of the flagellar export channel (20–30 Å) appears to be too small for the transport of well-folded proteins [3]. It is an open question whether flagellar chaperones can keep their target proteins in a partially unfolded, export-competent conformation. Our DSC experiments demonstrate that FliS binding does not interfere with the temperature induced unfolding of the compact central part of flagellin, it has no significant effect on the structural stability of the well-folded flagellin domains. Thus, the chaperone binding does not promote unfolding of FliC and does instead facilitate the formation of secondary structure in its chaperone binding region. While it is possible that flagellin subunits traverse the central channel of flagellar filaments as unfolded polypeptides, it is not the function of the chaperone to promote this by keeping the compact part of flagellin in an export-competent state.

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